

Comparative topical pheromonotropic activity of insect pyrokinin/PBAN amphiphilic analogs incorporating different fatty and/or cholic acid components☆

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Abstract

This study presents a comparison of the topical pheromonotropic activity in the tobacco budworm moth of a series of amphiphilic pseudopeptide analogs of the insect pyrokinin/PBAN peptide class incorporating fatty acids of varying chain lengths. While the C16 analog fails to penetrate the moth cuticle, and the C12 only moderately so, shorter chain analogs transmigrate the moth cuticle readily with decreasing cuticle-retention properties. A cholic acid analog topically induces twice the maximal pheromone titer of injected native hormone. From a pest management perspective, these non-aromatic hydrophobic components are expected to be more environmentally benign than benzenoid components previously used in topical insect peptide analogs. © 2001 Published by Elsevier Science Inc.

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1. Introduction

Insect neuropeptides are critically important bioregulators of a number of aspects of insect physiology and behavior [3,20]. Potent and specific as these molecular messengers are, neuropeptides in and of themselves are not suitable as pest insect control agents and/or tools for insect neuroendocrinologists due to their susceptibility to degradation by peptidases in the hemolymph and tissues and their polar nature that prevents them from penetrating the insect cuticle [12]. In recent work we have addressed the latter problem through the development of pseudopeptide analogs with amphiphilic character, which confers an ability to penetrate the hydrophobic insect cuticle and simultaneously maintains the water solubility necessary for them to re-emerge in the insect circulatory system and reach their target receptor sites [1,14,15,21,22]. Thus, modification of the peptide nature of the insect neuropeptides can make them amenable to use as

insect control agents employing traditional spray and bait station protocols for delivery.

Our research on topically active insect neuropeptide analogs has centered on the pyrokinin/PBAN family, which regulates a number of different physiological processes and is widespread among insect orders [13]. In orthopteran insects, such as locusts and cockroaches, the pyrokinins demonstrate myotropic activity on the gut and oviduct [4,19,20], while in Lepidoptera they induce sex pheromone biosynthesis [7,8,9,18], larval reddish-coloration and melanization [10], and in the silkworm moth they induce egg diapause [5]. In Diptera, they have been found to accelerate the pupariation process of the flesh fly [16,27]. Members of the family range in length from 8–34 amino acids, but all share the common C-terminal pentapeptide sequence FXPRL-amide, which retains at least some of the activity of the parent peptides in all of the physiological processes mentioned above [13]. The development of amphiphilic analogs of the pyrokinins began with this C-terminal active core, which already contains a component necessary for amphiphilic character, Arg, a highly charged and polar species. In one instance, the other component necessary for amphiphilic character, a highly hydrophobic species, was introduced via replacement of the phenyl ring of the core

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N-terminal Phe residue with the ball-shaped *o*-carborane moiety [15]. In other examples, the incorporation of hydrophobic character was accomplished by addition of organic acids containing aromatic groups to the N-terminus of the pentapeptide core sequence [1,14,21]. In each of these instances, the resulting amphiphilic analogs demonstrated an ability to transmigrate the cuticle of the tobacco budworm female moth, *Heliothis virescens*, and induce pheromone production about 15 min following topical application of an aqueous solution to the lateral abdominal cuticular surface. Interestingly, amphiphilic analogs containing larger and more hydrophobic aromatic groups demonstrated an ability to induce continuous production of high titres of pheromone over unnaturally long durations of greater than 18 h. The insect cuticle is composed of an apolar waxy layer atop a polar protein and chitin matrix and the prolonged pheromone production produced by some of the amphiphilics suggested that the cuticle can serve as a reservoir from which a slow release of analog can occur [14,21]. Indeed, experiments involving direct monitoring of analog passage through dissected pieces of *H. virescens* cuticle via HPLC demonstrate that some of these analogs do emerge from the cuticle in a slow steady rate following an initial burst of analog that occurs shortly after topical application. The ease with which an amphiphilic analog penetrated was dependent on the degree of apolarity and size of the hydrophobic component. As an example, while a total of 350 pmoles were recovered over 24 h from an initial application of 500 pmoles (70%) to isolated moth cuticle for the amphiphilic analog 6Pha-FTPRLa (1 phenyl ring), only 24 out of a possible 500 pmoles (5%) were recovered over a 24 h period for 9Fla-FTPRLa (2 phenyl groups within a broad fluorene ring system). On the other hand, while penetration of the 6Pha analog ceased after 6 h, significant quantities of the 9Fla analog were emerging up to 20 h following application to isolated moth cuticle. Thus, while significantly larger overall quantities of the smaller, less hydrophobic component of the 6Pha analog penetrated the isolated cuticle, the hydrophobic fluorene-containing 9Fla analog demonstrated superior time-release characteristics [22].

In this investigation, we explore the effects that non-aromatic hydrophobic components of different apolarity and size have on the ability of pseudopeptide analogs of the pyrokinin/PBAN active-core sequence to penetrate the cuticle and induce pheromone production in the tobacco budworm moth following topical application. Specifically, the study focuses on a comparison of the topical pheromotropic activity of pyrokinin/PBAN pseudopeptide analogs containing fatty acids of different chain lengths (C2, C5, C8, C10, C12 and C16) as well as cholic acid, which features the ring system of cholesterol. In addition, we explore the topical activity of an amphiphilic pyrokinin/PBAN analog containing two different classes of hydrophobic components, both a carboranyl group and a fatty acid (C5). A demonstration that fatty acids and/or cholic acid can be utilized in the development of topical pyrokinin/PBAN an-

alogs would serve to expand the available non-aromatic hydrophobic components beyond the carboranyl group, all of which would be expected to be more environmentally friendly than pseudopeptide analogs containing benzenoid components.

2. Method

2.1. Pseudopeptide synthesis

The pyrokinin amphiphilic pseudopeptides were synthesized by condensation of the fatty acid components (either acetic [C2], valeric [C5], caprylic [C8], caproic [C10], lauric [C12] or palmitic [C16]; purchased from Aldrich Chemical Co., Milwaukee, WI) or cholic acid (purchased from Alfa-Aesar, Ward Hill, MA) with the peptide-resin complex Phe-Thr-Pro-Arg(Pmc)-Leu-Rink Amide (synthesized via Fmoc solid phase technology according to previously described procedures [11,15]) using one equivalent of a 1,3-diisopropylcarbodiimide/1-hydroxy-7-azabenzotriazole mixture in dimethyl sulfoxide for 4 h according to previously described procedures [15].

Racemic *o*-Cba was obtained as described recently [17] using the methodology of Wyzlic et al. [26]. The preparation of the Fmoc derivative of the carborane-containing amino acid [(±)-*N*-(fluorenylmethoxycarbonyl)-*o*-carboranylalanine] was as follows. To an ice-cold suspension of (±)-*o*-CBA HCl salt (1.75 g, 6.53 mmol) and acetonitrile (22 ml) was added triethylamine (1.80 ml, 12.9 mmol) followed by Fmoc-succinimide (2.21 g, 6.53 mmol). After stirring the reaction mixture at 0°C for 3 h, then room temperature for 3 h, it was concentrated under reduced pressure below 35°C. The brown residue was partitioned between 1N aq. HCl solution (25 ml) and ethyl acetate (30 ml). The phases were separated and the aqueous phase extracted with ethyl acetate (2 × 15 ml). The combined organic extracts were washed with saturated NaCl solution then concentrated under reduced pressure. The oily residue was purified by silica gel column chromatography using gradient elution (1 to 20% methanol in chloroform) to yield 2.26 g (76%) of the title product as a white solid. Mp.: 135–137°C.

¹H NMR (300 MHz, CDCl₃) δ 7.29–7.77 (m, 8H), 5.80(broad s, 1H), 3.90–4.5(m, 5H), 2.72 (m, 2H), 1.05–3.20(broad m, 10H). ¹³C-NMR (75 MHz, CDCl₃) δ 174.8, 156.0, 143.7, 141.2, 127.7, 127.1, 125.1, 120.0, 73.8, 66.8, 60.6, 54.8, 47.1, 39.4. FAB: 478.7 ([M+Na]⁺) (within the expected B10 isotope cluster pattern) [C₂₀H₂₇B₁₀NO₄ calcd. (M+Na)⁺: 478.29].

The pyrokinin pseudotetrapeptide analog was synthesized by condensation of Cbp (carboranylpropanoate) to the Thr-Pro-Arg(Pmc)-Leu-Rink amide resin complex with one equivalent of a 1,3-diisopropylcarbodiimide/1-hydroxy-7-azabenzotriazole mixture in dimethyl sulfoxide for 4 h according to previously described procedures. The Thr-Pro-

Arg(Pmc)-Leu-rink amide resin complex was synthesized via Fmoc methodology as previously described [15]. Protected amino acid starting materials were purchased from Advanced Chemtech (Louisville, KY). The pseudopeptide crude products were cleaved from the resin complex by stirring with a mixture of trifluoroacetic acid (95%), anisole (5%), thioanisole (4%), and 1,2-ethanedithiol (1%) for 1 h at RT. The resin was filtered and volatile reagents were removed in vacuo on a Savant Speed Vac concentrator at 40°C.

The products were purified on a Waters C18 Sep Pak cartridge and a Delta Pak C18 reverse-phase column at ambient temperature on a Waters Model 510 HPLC controlled with a Millennium 2000 chromatography manager system (Waters, Milford, MA) with detection at 214 nm. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: initial solvent consisting of 20% B was followed by Waters linear program 6–1005B to 100% B over 40 min; flow rate: 2 ml/min. Retention times on C18 column: **C2-FTPRLa**, 11.25 min; **C5-FTPRLa**, 17.25 min; **C8-FTPRLa**, 23.5 min; **C10-FTPRLa**, 29.0 min; **C12-FTPRLa**, 35.25 min; **C16-FTPRLa**, 37.5 min; **Cholic-FTPRLa**, 21.0 min; **Valeric-CbAla-TPRLa**, 24.0 min. Several of the amphiphilic analogs required further purification on a Waters Protein Pak 125 column. Solvent A = 95% aqueous acetonitrile containing 0.01% TFA; solvent B = 50% acetonitrile containing 0.01% TFA. Conditions: 100% A isocratic for 4 min followed by linear program to 100% B over 8 min. Retention times on Protein Pak 125 column: **C8-FTPRLa**, 13.5 min; **C10-FTPRLa**, 13.6 min; **Cholic-FTPRLa**, 14.25 min; **Valeric-CbAla-FTPRLa**, 6.25 min (containing both diastereomers). The pure peptides were analyzed and quantified via amino acid analysis. Each peptide sample was purged with N₂ and the peptide hydrolyzed with vapor-phase HCl for 24 h at 105°C. Precolumn derivatization and HPLC analysis was accomplished by the standard PicoTag method supplied by Waters (Milford, MA). The observed amino acid ratios were as expected for each analog: **C2-FTPRLa** - F[1.0], L[1.0], P[1.1], R[1.0], T[1.1]; **C5-FTPRLa** - F[1.0], L[1.0], P[1.0], R[1.1], T[1.1]; **C8-FTPRLa** - F[1.0], L[1.0], P[1.0], R[0.9], T[1.0]; **C10-FTPRLa** - F[1.0], L[1.0], P[0.9], R[1.1], T[1.0]; **C12-FTPRLa** - F[1.0], L[1.0], P[1.0], R[1.0], T[1.0]; **C16-FTPRLa** - F[1.0], L[1.0], P[0.9], R[1.1], T[1.1]; **Cholic-FTPRLa** - F[1.0], L[1.0], P[1.0], R[0.9], T[0.8]; **Valeric-CbAla-TPRLa** - L[1.0], P[1.1], R[1.0], T[0.9]. Fast atom bombardment (FAB) mass spectra were obtained by adding 10 µg of analog sample to glycerol (1.5 µl) on a copper probe, followed by bombardment with 8 kV Xe atoms on a Kratos MS-50 mass spectrometer (Kratos, Manchester, UK). The structural identity and a measure of purity of the analogs was confirmed by the presence of the following molecular ions (MH⁺) and the absence of contaminant peaks: **C2-FTPRLa**, 674.4 [calc MH⁺: 674.4]; **C5-FTPRLa**, 716.4 [calc MH⁺: 716.4]; **C8-FTPRLa**, 758.5 [calc MH⁺: 758.4];

C10-FTPRLa, 786.5 [calc MH⁺: 786.4]; **C12-FTPRLa**, 814.6 [calc MH⁺: 814.4]; **C16-FTPRLa**, 870.5 [calc MH⁺: 870.4]; **Cholic-FTPRLa**, 1022.6 [calc MH⁺: 1023.4]; **Valeric-CbAla-TPRLa**, 784.6 (within the expected B10 isotope cluster pattern)[calc. MH⁺: 784.59].

2.2. *Heliothis virescens*

Pupae of *H. virescens* were obtained from a laboratory colony maintained at the Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, in Gainesville, FL. The pupae were separated by sex and the males were discarded. After eclosion, the females were held in 4-liter cages and provided with cotton wicks soaked with 5% sucrose solution. The insects were held at 25 ± 2°C, 65 ± 3% relative humidity with a 12:12 (L:D) photoperiod. Virgin females were used for bioassays 2 and 3 days after eclosion during the 7–8th hour of the photophase when endogenous levels of pheromone are very low [24].

2.3. Pheromonotropic assay (injection)

Pheromone biosynthesis activating neuropeptide from *H. zea* (Hez-PBAN) and the pyrokinin amphiphilic pseudopeptides were dissolved in physiological saline [2]. Female *H. virescens* insects were injected through the side of the third abdominal segment using a 26-ga needle with 10 µl of the above solutions. Females were injected with amounts of pseudopeptides ranging from 0.005–1000 pmol or with 5 pmol of Hez-PBAN, as a positive control or just saline. After 1 h of incubation, the terminal abdominal segments, which contain the pheromone gland, were excised and placed in 10 µl of hexane containing 10 ng each of internal standards (octadecane and nonadecane) used for quantitation and calculation of relative retention indices. The pheromone gland extracts were analyzed for the amount of (Z)-11-hexadecenal (Z11-16:AL), the major sex pheromone component of *H. virescens* [6] by capillary gas chromatography. Hewlett-Packard 5890 gas chromatographs were equipped with splitless injectors, flame ionization detectors, and 30 m × 0.25 mm (i.d.) fused silica SPB10 (polar) or SPB1 (apolar) columns (Supelco). Conditions of chromatography were as follows: initial temperature = 60°C; injector operated in the splitless mode for 0.5 min; oven temperature increased by 30°C/min after 1 min; final temperature = 190°C (SPB10) or 200°C (SPB1); helium carrier gas at a linear flow velocity of 18 cm/s. A Nelson Analytical 3000 acquisition system (Cupertino, CA) was used to collect and analyze the data [25]. The amounts of pheromone present in extracts of the pheromone glands of females injected with various amounts of pseudopeptides were compared with that produced by females injected with 5 pmol of Hez-PBAN (or with just saline) during that particular bioassay. After all the bioassays were completed, the amount of pheromone produced by each insect was calculated as a percentage of the amount of pheromone

Table 1

Pheromonotropic^a Activity of Pyrokinin Amphiphilic Analogs Containing Fatty Acid Components via Topical Application and Injection

Chain Length	Maximal Response ^b (pmoles)	Topical ED ₅₀ (pmoles)	Injected ED ₅₀ (pmoles)
C2	25	13 (3–47) ^d	0.007 (0.004–0.1) ^d
C5	100	47 (26–82) ^d	0.08 (0.05–0.1) ^d
C8	102 (175%) ^c	66 (44–99) ^d	0.28 (0.17–0.47) ^d
C10	102	88 (54–143) ^d	5.1 (3.4–4.6) ^d
C12	2000 (70%) ^c	1616 (667–3913) ^d	9.8 (6.7–14.4) ^d
C16	inactive	—	43 (26–68) ^d
C5-CbAla ^e	400	281 (186–424) ^d	0.02 (0.008–0.06) ^d

^a Values represent the mean amounts determined from analysis of eight replicates/dose (\pm SEM). Contents of pheromone gland analyzed 1 hr following application.

^b Topical dose of analog eliciting maximal pheromone production relative to 5 pmol PBAN via injection.

^c The figure in parentheses represents maximal pheromone titer as a percentage of injected PBAN, in which the difference is statistically significant ($P < 0.01$).

^d Figures in parentheses represent lower and upper 95% confidence limits.

^e C5-CbAla represents a C5 fatty acid attached to carboranylalanine.

produced by females treated with Hez-PBAN. Data were analyzed using one-way ANOVA and *t* test with Statview®. Variability was reported as SEM.

2.4. Pheromonotropic assay (topical)

The amphiphilic pseudopeptide analogs were dissolved in water and solutions at concentrations of between 0.1–2000 pmol/ μ l were prepared. The cuticular scales on the ventral surface of the abdomen were removed by gently rubbing the abdomen on cellophane tape. After the scales were removed, 1 μ l of pseudopeptide solution was applied to the surface of the cuticle. The insects were allowed to incubate 1 h and then the pheromone gland was excised and the amount of pheromone determined by the method described above for the injected insects.

3. Results

Before evaluation of the topical activity of the amphiphilic analogs, the pheromonotropic activity was determined

via injection to ascertain intrinsic biologic activity separate from their interactive behavior with the moth cuticle. A number of the analogs demonstrated pheromonotropic activity with ED₅₀'s in the range of 0.007 to 0.28 pmoles, including the C2, C5, and C8 fatty acid analogs and the hybrid analog containing both a C5 fatty acid and a carboranyl component (Table 1). An ED₅₀ of 0.1 pmoles, also within this range, had been previously [15] reported for the analog containing only a carboranyl component (Table 2). The cholic acid analog demonstrated a similar ED₅₀ of 0.29 pmoles (see Table 2). Pseudopeptide analogs containing the longer fatty acid chain lengths of C10 and C12 both demonstrated less potent ED₅₀ levels of 5 and 9.8 pmoles, respectively. The C16 pseudopeptide analog was least potent of all with an ED₅₀ of 43 pmoles (see Table 1).

Following the injection trials, the pseudopeptides were applied to the descaled abdominal surface of *Heliothis virescens* at various doses in 1 μ l of aqueous solution, allowed to incubate for 1 h and the pheromone gland was then excised, extracted and the extract analyzed to determine the amount of pheromone produced. The pseudopeptide series

Table 2

Pheromonotropic^a Activity of Pyrokinin Amphiphilic Analog Containing Non-Fatty Acid, Non-Aromatic Components via Topical Application and Injection

Hydrophobic Component ED ₅₀	Maximal Response ^b (pmoles)	Topical ED ₅₀ (pmoles)	Injected (pmoles)
Cholic Acid	55 (200%)	42 (28–62) ^c	0.29 (0.02–1.8) ^d
Cbp ^e [15]	60	25	0.1

^a Values represent the mean amounts determined from analysis of eight replicates/dose (\pm SEM). Contents of pheromone gland analyzed 1 hr following application.

^b Topical dose of analog eliciting maximal pheromone production relative to 5 pmol PBAN via injection.

^c The figure in parentheses represents maximal pheromone titer as a percentage of injected PBAN, in which the difference is statistically significant ($P < 0.01$).

^d Figures in parentheses represent lower and upper 95% confidence limits.

^e Cbp represents carboranylpropanoate.

incorporating fatty acids of varying carbon chain length at the N-terminus of the pyrokinin core pentapeptide provided a very interesting set of results. The pseudopeptide containing a C2 acyl group (acetate) elicited a maximal pheromone titer (100% is equivalent to 5 pmoles of Hez-PBAN injected) with a topical application of only 25 pmoles ($ED_{50} = 7$ pmoles). Pseudopeptides containing C5 (valerate) and C8 (caprylate) acyl groups elicited a 100% pheromone response with topical applications of 100 pmoles (both with an $ED_{50} = 50$ pmoles). Interestingly, the C8 pseudopeptide elicited a statistically significant supra-maximal response of 175% at a topical dose of 250 pmoles. The C10-containing (caproate) pseudopeptide elicited a 100% maximal response at about 100 pmoles with a topical ED_{50} of 65 pmoles. The pseudopeptide incorporating a C12 (laurate) acyl group demonstrated a clear drop-off in topical activity, unable to produce a 100% maximal pheromonotropic response even up to 2000 pmoles, the highest dose tested. The C12 analog elicited only a 70% maximal response at 2000 pmoles and could produce a response 50% of that of the natural Hez-PBAN at a topical dose of 1500 pmoles. The final member of this fatty acid series, incorporating a C16 (palmitate) acyl group, failed to demonstrate any topical activity at all, with no significant difference in pheromone titer from control animals even up to a dose of 2000 pmoles (Table 1). To determine whether this C16 analog retained any pheromonotropic activity at all, the experiment was repeated via injection. The results demonstrated that the C16 pseudopeptide had pheromonotropic activity at an ED_{50} of 43 pmoles, although no response could be detected at doses of 15 pmoles or below. A pseudopeptide analog incorporating a cholic acid group, which features the ring system of cholesterol, demonstrated high topical activity. The cholic analog could elicit a 100% response at 55 pmoles ($ED_{50} = 42$ pmoles), and at a dose of 250 pmoles could produce a statistically significant supramaximal pheromone response of 200% of that of the natural Hez-PBAN, making it the most efficacious topical analog tested (Table 2). The amphiphilic pyrokinin pseudopentapeptide analog incorporating two different classes of hydrophobic groups, a carboranylalanine residue and an N-terminal C5 (valerate) group (**Vla-CbAla-FTPRLa**; Vla = valerate; CbAla = carboranylalanine), elicited a 100% pheromone response at a dose of 400 pmoles ($ED_{50} = 281$ pmoles). The additional presence of a valerate group changed the degree of topical activity markedly, as the pseudotetrapeptide analog **Cbp-TPRLa** (Cbp = carboranylpropanoate), lacking an N-terminal amino group, elicited a 100% response at a dose of only 60 pmoles and with a more potent topical ED_{50} of 25 pmoles [15].

4. Discussion

The series of pyrokinin pseudopentapeptides incorporating fatty acids of varying chain length allows for a unique

investigation of the effects of the hydrophobic component on topical pheromonotropic activity in the tobacco budworm moth, *Heliothis virescens*. Each member of the fatty acid amphiphilic pyrokinin analog series was evaluated on the quantity of the pheromone titer produced at various doses 1 h after topical application. Under these short-duration conditions, the results clearly established that the C2 (acetate) amphiphilic analog (**Ac-FTPRLa**) demonstrated the highest topical potency, with an ED_{50} of 13 pmoles, and reached the maximal level of pheromone production of injected natural Hez-PBAN at only 25 pmoles. Amphiphilic analogs containing fatty acid chain lengths of C5, C8 and C10 (**Vla-FTPRLa**, **Cpa-FTPRLa** and **Cap-FTPRLa**) elicited topical pheromone production with similar potencies (ED_{50} 's of 47, 66 and 88 pmoles, respectively), and all three reached a 100% level of pheromone production following application of about 100 pmoles to the cuticular surface. The C8 amphiphilic analog proved to be the most efficacious among all of the fatty acid-containing amphiphilic analogs, which at a topical dose of 250 pmoles produced a statistically significant 250% of the maximal titer of injected natural Hez-PBAN. Both topical potency and efficacy began to erode as the chain length of the fatty acid component was increased to C12 (**Lac-FTPRLa**), which demonstrated a 30-fold reduction in potency, requiring 1500 pmoles to reach 50% of the maximal pheromone titer of Hez-PBAN. Even up to the highest dose used in this study, 2000 pmoles, the C12 amphiphilic analog could elicit only 70% of the maximal pheromone titer of the natural neuropeptide Hez-PBAN. The difference between the topical activity of the C12 and C10 pseudopeptide analogs is clearly due to their respective cuticle-retention characteristics, because both analogs demonstrate similar intrinsic biologic activity as determined via injection (injected ED_{50} values of 5.1 and 9.8 pmoles, respectively). The final member of the fatty acid amphiphilic analog series, containing a C16 hydrophobic component (**Pal-FTPRLa**) proved to be ineffective as a topical pheromonotropin. No statistically significant quantities of pheromone could be detected even up to the highest dose of 2000 pmoles, despite an ability by the C16 analog to elicit pheromone production when administered via injection. Clearly, the C16 (palmitate) analog failed to appreciably penetrate the cuticle during the time frame of the study. This is likely due to its affinity to the epicuticular waxes which contain a large palmitate component. The cuticle apparently serves as a reservoir for the palmitate analog from which it fails to emerge into the hemolymph. The topical activity results from this series of fatty acid-containing analogs indicate that this retentive effect diminishes as the chain length becomes progressively shorter.

Attachment of cholic acid, which features the ring system of cholesterol, to the pyrokinin core pentapeptide leads to a highly efficacious topical analog. At 250 pmoles, it produced the highest titer of pheromone of any analog so far examined, a statistically significant 200% of the maximal titer of injected Hez-PBAN. It also demonstrates high po-

tency, reaching 50% of the maximal pheromone titer of PBAN at a dose of 55 pmoles ($ED_{50} = 42$ pmoles).

Addition of more than one class of hydrophobic component, as in the pseudopentapeptide analog **Vla-CbAla-TPRLa** (Vla = valerate; CbAla = carboranylalanine), appears to lead to an expected increase in cuticle-retention and a reduction in topical pheromonotropic potency when monitored at the 1 h post-application time period. The topical ED_{50} is reduced by almost an order of magnitude over the pseudotetrapeptide **Cbp-TPRLa** (Cbp = carboranyl propanoate). The increase in cuticle retention behavior of **Vla-CbAla-TPRLa** is underscored by the fact that this hybrid fatty acid-carboranyl analog is actually five-fold more potent than the simple carboranyl analog **Cbp-TPRLa** when administered via injection.

In closing, the present results have demonstrated that fatty acids and cholic acid can be successfully used as hydrophobic components in the development of amphiphilic analogs of insect neuropeptides capable of penetrating the cuticle of moths. These classes of hydrophobic hydrocarbons expand the available non-aromatic hydrophobic components beyond the carboranyl group. All of these non-aromatic hydrophobic components would be expected to lead to more environmentally friendly pseudopeptide analogs of insect neuropeptides than benzenoid components used in previous studies. While the acetate (C2) amphiphilic analog proved to have the highest potency and most facile cuticle-penetrability properties of the fatty acid analog series, it is not likely to be the ideal hydrophobic component for topical insect neuropeptide analog candidates. In earlier studies on amphiphilic analogs containing aromatic acyl components, we have demonstrated that they exhibit potent pheromonotropic effects when injected into adult female moths and that they can function effectively when applied topically in aqueous solution. However, some of these analogs were active for only 4–8 h following topical application. Short periods of bioactivity can limit the usefulness of such pseudopeptides for the development of new pest insect management methods because such strategies require prolonged physiological and behavioral responses following exposure [23]. Thus the apolar insect cuticle matrix should be harnessed as a reservoir from which amphiphilic pseudopeptide analogs, designed with an appropriate balance of polar and apolar components, can be slowly released into the hemolymph. For instance, earlier studies demonstrated that analogs containing larger and more hydrophobic aromatic components of 2 or more phenyl rings could elicit unnaturally prolonged and continuous production of high titers of pheromone stretching out to 20 or more hours after application [14,22].

The inability of the palmitate (C16) amphiphilic analog to penetrate the cuticle of *H. virescens* and the relatively poor topical activity demonstrated by the C12 analog indicates that the ideal fatty acid chain length must be shorter than 12 carbons. The advisability of appropriate cuticle-retention properties conducive to slow release of pseu-

dopeptide analogs into the hemolymph suggests that the fatty acid chain length should exceed 2 carbons. From the results presented in this study, the ideal chain length for the hydrophobic component of an amphiphilic pyrokinin analog in *H. virescens* would likely be between C8 and C10, inclusive. Future studies of the topical pheromonotropic activity of these analogs conducted over longer time periods, in addition to studies aimed at directly monitoring the extent of analog penetration through isolated cuticle, can delineate those analogs with ideal cuticle-retention properties. Each of these studies will have to be repeated for different insects as cuticle composition and density vary considerably from one insect type to another [22,23]. Furthermore, pest management strategies based on mimetic analogs of insect neuropeptides may best be served by addition of both an analog with fast penetration characteristics to elicit an immediate physiological effect and an analog with slow release properties to maintain a prolonged physiological effect.

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